DnaA and ORC

Scholefield, Graham; Veening, Jan-Willem; Murray, Heath

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Review

DnaA and ORC: more than DNA replication initiators

Graham Scholefield[1], Jan-Willem Veening[2] and Heath Murray[1]

[1] Centre for Bacterial Cell Biology, Institute for Cell and Molecular Biosciences, Newcastle University, Newcastle Upon Tyne, NE2 4AX, UK
[2] Molecular Genetics Group, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands

Mutations in DNA replication initiator genes in both prokaryotes and eukaryotes lead to a pleiotropic array of phenotypes, including defects in chromosome segregation, cytokinesis, cell cycle regulation and gene expression. For years, it was not clear whether these diverse effects were indirect consequences of perturbed DNA replication, or whether they indicated that DNA replication initiator proteins had roles beyond their activity in initiating DNA synthesis. Recent work from a range of organisms has demonstrated that DNA replication initiator proteins play direct roles in many cellular processes, often functioning to coordinate the initiation of DNA replication with essential cell-cycle activities. The aim of this review is to highlight these new findings, focusing on the pathways and mechanisms utilized by DNA replication initiator proteins to carry out a diverse array of cellular functions.

Introduction

It has long been appreciated that DNA synthesis needs to be tightly regulated by replication initiator proteins (DnaA in bacteria and ORC [origin replication complex] in eukaryotes) during the cell cycle and coordinated alongside the availability of resources to ensure proper duplication and segregation of the genome. The critical role of replication initiators has led to their intensive study and, while the mechanism by which DnaA acts at the origin of replication during initiation is broadly understood, the mechanism of action of ORC has remained more elusive (Box 1).

Work on bacterial and eukaryotic DNA replication initiator proteins has revealed that, in addition to their essential activity required for DNA synthesis, they affect diverse processes including chromosome segregation, cytokinesis, cell cycle regulation and gene expression [1–3]. However, for many years it was unclear whether these phenotypes were the indirect consequence of impaired DNA synthesis, or whether DNA replication initiator proteins participated directly in these various pathways. A growing body of evidence supports the idea that DNA replication initiator proteins are directly involved in many of these cellular activities (Figure 1). In this review, we discuss studies on both bacterial and eukaryotic model organisms that have led to our understanding of the diverse activities of DNA replication initiator proteins. The conserved utilization of initiator proteins in both simple and complex organisms for a broad range of cellular functions suggests that initiator proteins act as a critical hub to integrate DNA synthesis with the cell cycle.

The role of DNA replication initiator proteins in chromosome organization and segregation

All cells must replicate and segregate their chromosomes to ensure faithful inheritance of their genetic information. DNA replication is a tightly regulated event that requires DNA replication initiator proteins first become activated in order to commence DNA synthesis, followed by their inactivation to guarantee that DNA replication occurs only once per cell cycle (Box 1). Because of this cycle of activation/deactivation, and because DNA replication must occur before chromosome segregation, DNA replication initiator proteins would make logical candidates to connect these processes.

To facilitate accurate chromosome segregation in eukaryotic cells during mitosis, replicated chromosomes are held together through the process of sister-chromatid cohesion until the metaphase to anaphase transition when they are separated by microtubules and associated motor proteins [4]. Genetic studies in both budding yeast and fruit flies showed that *orc* mutants (*orc2-1, orc5-1* and Δ*orc6*) inhibit mitosis and cause a synthetic growth defect when combined with mutations in genes that affect sister-chromatid cohesion [5–7]. Recent findings suggest that ORC directly affects chromosome segregation independent of DNA replication initiation by playing two distinct roles in sister-chromatid cohesion and also by regulating protein complexes required for microtubule organization (Figure 2).

Following DNA replication, sister chromatids are linked together by the cohesin complex, a multisubunit ring that encircles the paired DNA molecules [4]. Several studies have indicated that ORC participates in sister-chromatid cohesion through the localization of cohesin complexes. Orc5 is required for recruitment of cohesin to centromeres in the fission yeast *Schizosaccharomyces pombe* and ORC is required for cohesin loading in *Xenopus* egg extracts [8,9]. Moreover, ORC colocalizes with cohesin in the fruit fly *Drosophila melanogaster* [10]. In the cases of *S. pombe* and *D. melanogaster,* it appears that proper cohesin localization occurs under conditions when replicative helicase loading is inhibited, indicating that the involvement of ORC in this pathway is separable from its activity in initiating DNA replication. It remains to be determined whether ORC recruits cohesin via a direct protein–protein

Corresponding author: Murray, H. (heath.murray@newcastle.ac.uk).

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Box 1. Initiation of DNA replication in bacteria and eukaryotes

The bacterial DnaA and the eukaryotic origin recognition complex (ORC, which is composed of six subunits referred to as Orc1–Orc6) are functional homologues and share several features and activities (Figure I). First, all initiators contain specific motifs that facilitate DNA binding. While DnaA recognizes specific DNA sequences within identified replication origins using its C-terminal helix-turn-helix (HTH) motif, the localization of ORC at replication origins in higher organisms appears to be less dependent on sequence and is thought to be mediated through potential DNA-binding motifs found within multiple ORC subunits. Second, DnaA and ORC are ATP-binding proteins that contain homologous AAA+ motifs (present in five of the six Orc proteins). Functional and structural studies indicate that both DnaA and ORC undergo ATP-dependent conformational changes that activate the proteins to allow the initiation of DNA replication. Third, initiator proteins assembled at replication origins load the replicative helicases required for bidirectional DNA replication elongation. In bacteria, the N-terminal domain of DnaA interacts directly with the helicase to load the enzyme onto DNA, whereas in eukaryotes ORC recruits two additional proteins, Cdc6 and Ctd1, that are required for helicase loading. Lastly, both initiators are inactivated following DNA replication initiation to ensure that genome duplication occurs only once per cell cycle. DnaA can be inactivated through hydrolysis of its bound ATP, while ORC inactivation is more complex and is thought to involve ATP hydrolysis, loss of Cdc6/Cdt1 binding and/or phosphorylation. Please see [48,55–57] for excellent reviews with more detailed information regarding the regulation and mechanisms of DNA replication initiator proteins in DNA synthesis.

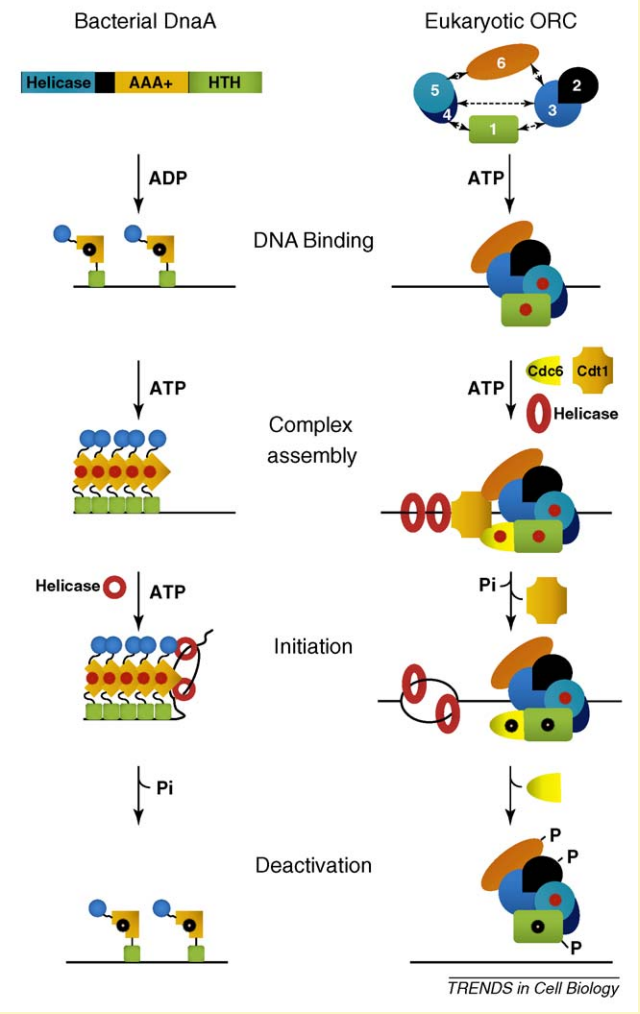

Figure I. Initiation of replication in bacteria and eukaryotes.

interaction, or whether additional factors are required. We note that ChIP-chip analysis has shown that cohesin is enriched at locations of convergent transcription, and it has been proposed that transcription complexes might act as road blocks that restrict cohesin movement [11]. It could be that DNA replication initiation complexes act in an analogous manner to affect cohesin localization. Moreover, because DNA replication is initiated from multiple origins along eukaryotic chromosomes, initiation complexes could help to distribute cohesin along the substrate DNA.

Bacterial condensin complexes, which are structurally related to cohesin complexes, are required for accurate chromosome organization and segregation [12,13]. Interestingly, recent work in both *Bacillus subtilis* and *Escherichia coli* has found that condensin complexes are enriched at DNA replication origins in both of these distantly related bacteria (Gram-positive and Gram-negative, respectively), indicating that this region plays the role of an 'organizational centre' from which the condensin complexes act [14–16]. This observation raises the possibility that bacterial initiator proteins play a role in condensin complex localization. If so, then this conservation would argue that the interaction of cohesin/condensin family members with DNA replication initiator proteins is crucial for proper chromosome organization in both bacteria and eukaryotes.

Along with its role in the cohesin-dependent pathway of sister-chromatid cohesion, ORC facilitates the conjunction of sister chromatids independently of cohesin complex recruitment. In *S. cerevisiae*, sister-chromatid cohesion is disrupted following depletion of Orc2 during late G1 phase (i.e., after proper loading of the replicative helicase), despite the fact that cohesin complexes are loaded onto sister chromatids [17]. Moreover, ectopic integration of DNA-binding sequences recognized by ORC was sufficient to establish sister-chromatid cohesion in the absence of cohesin, firmly establishing that this ORC-mediated pathway plays an independent role in chromosome segregation during mitosis. However, further work is needed to determine whether ORC directly mediates sister-chromatid cohesion or whether additional factors are required.

Along with role(s) in chromosome segregation, which occur on the substrate DNA in the nucleus, it was recently revealed that ORC proteins regulate centrosome duplication within the cytoplasm. The mammalian centrosome consists of two centrioles that act during the transition of metaphase to anaphase to organize the microtubules required to separate sister chromosomes before cell division (Figure 2) [18]. Centrosomes are disengaged and licensed for duplication during G1 phase before being replicated during S phase and then re-engaged, a process requiring several regulatory proteins. Studies in human cells have shown that the Orc1-4 proteins localize to centrosomes and that depletion of Orc1 leads to centrosome over-replication [19,20]. Further work demonstrated that the activity of Orc1 is dependent on two cyclin proteins: Cyclin A promoted Orc1 localization to centrosomes, whereupon Orc1 inhibited Cyclin E-dependent re-duplication of the centrosomes [19]. Future investigations should elucidate whether Orc subunits interact directly with the cyclins to regulate centrosome duplication, and whether this ORC

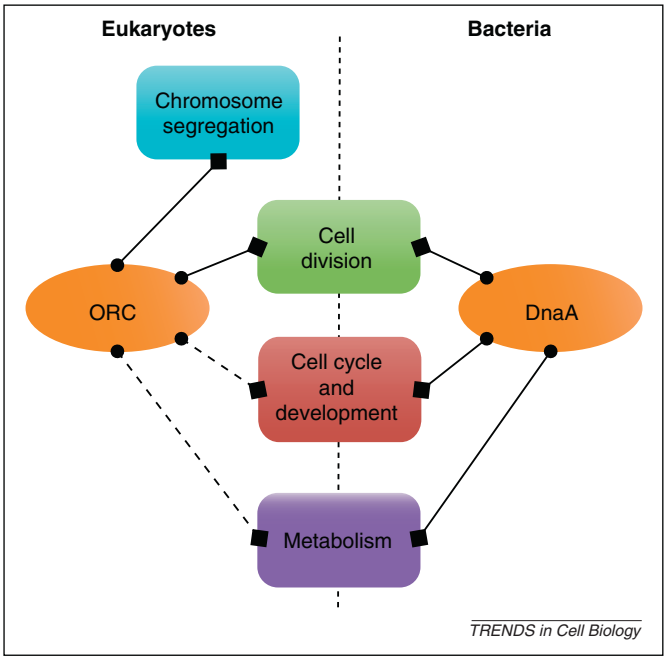

Figure 1. Summary of the potential roles played by DNA replication initiator proteins beyond DNA synthesis. Unbroken lines indicate that replication initiator proteins play direct roles in cellular processes. Broken lines indicate that the connection between the initiator protein and the cellular activity remains speculative.

activity is conserved in other eukaryotic cells. Importantly, human Orc1 is phosphorylated following DNA replication initiation, leading to the protein being exported from the nucleus [21]. This regulation of Orc1 localization could provide a critical link between the processes of DNA replication initiation and chromosome segregation during mitosis in higher eukaryotic cells.

DNA replication initiator proteins as regulators of cell division

Accurate chromosome segregation also requires the coordination of DNA synthesis with cell division and in most cells cytokinesis occurs after chromosome replication and segregation are complete. Defects in the regulation of cell division can lead to aneuploidy, polyploidy and DNA damage.

In the Gram-positive bacterium *B. subtilis*, DnaA plays a direct role coordinating DNA replication with cell division through transcriptional regulation of *ftsL*. FtsL is an essential membrane-bound protein that is required for recruitment of the cell division machinery [22]. FtsL is intrinsically unstable and is targeted by several regulatory proteases, leading to the protein being short-lived *in vivo* [23]. Due to its instability, the pool of FtsL must be constantly replenished at the level of gene expression, and reduced transcription of *ftsL* leads to a rapid block in cell division. Recent work has shown that *ftsL* transcription is repressed when DNA replication is perturbed (i.e., during replication stress) leading to a block in cell division [24,25]. Following replication stress, DnaA becomes enriched around the *ftsL* promoter region and causes transcriptional repression of *ftsL* leading to the inhibition of cell division. Sequence analysis of the *ftsL* promoter region revealed the presence of specific binding sites for DnaA, although the mechanisms of DnaA enrichment and transcriptional repression have not been determined. Interestingly, a comparison of the *ftsL* promoter regions from several diverse bacteria revealed that the presence of DnaA boxes is highly conserved, indicating that this regulatory pathway is crucial for bacterial physiology. Thus, under conditions when bacterial chromosome replication is compromised, the DNA replication initiator protein stalls cell division via transcriptional repression of *ftsL* to avoid fatal missegregation of the genome.

In addition to negative regulation of cell division, the DNA replication initiator protein coordinates DNA replication with cell division by activating the transcription of essential cell division genes in certain bacterial species. In the Gram-negative bacterium *Caulobacter crescentus,* DnaA is degraded at the end of each cell cycle and must be synthesized prior to each new round of DNA replication. In *Caulobacter*, DnaA also activates transcription of the essential cell division protein *ftsZ*, a tubulin homologue that acts as a scaffold protein for the assembly of the cell division machinery [26,27]. Further analysis demonstrated the presence of functional DnaA-binding sites within the *ftsZ* promoter region, indicating that activation by DnaA is likely to be direct, although again the mechanism of transcriptional regulation has not been determined. Thus, *Caulobacter* facilitates accurate chromosome inheritance by activating the expression of an essential cell division gene only under conditions when DNA replication is also set to occur.

In bacteria, DNA replication and cell division are often intimately linked, with cytokinesis occurring soon after chromosome replication and segregation are complete. In eukaryotes, however, DNA replication is temporally separated from cell division. Despite this difference recent genetic and biochemical studies have provided evidence that, in fact, regulation of cell division by DNA replication initiator proteins might also occur in eukaryotes. Knockdown of Orc6 using RNA interference causes cell division defects in both *Drosophila* and human cells [28,29]. In *Drosophila*, Orc6 interacts directly with the septin Pnut, a filament-forming GTPase that is essential for cytokinesis and is thought to mediate its activity by functioning as a protein scaffold [30]. In the presence of GTP, Orc6 stimulates the GTPase activity of the septin complex and causes filament disassembly, whereas in the absence of GTP, Orc6 stimulates septin filament formation. Sequence analysis of Orc6 homologues has revealed that these proteins are composed of a conserved N-terminal domain and a variable C-terminal domain [31]. Pnut interacts with the C-terminal domain of Orc6, suggesting that this region of the initiator protein might have evolved distinct functions within different species. Importantly, we note that *Drosophilia* ORC is phosphorylated *in vivo* and is a substrate for cyclin-dependent kinases *in vitro* [32], suggesting a potential regulatory link between DNA replication and cytokinesis in eukaryotic cells. We anticipate that future work regarding the role of Orc6 regulating Pnut activity will further our understanding of the specific activities of Orc and septin proteins during cell division.

DNA replication initiator proteins as cell cycle and developmental regulators

In the previous sections we have considered roles of DNA replication initiator proteins during two specific activities

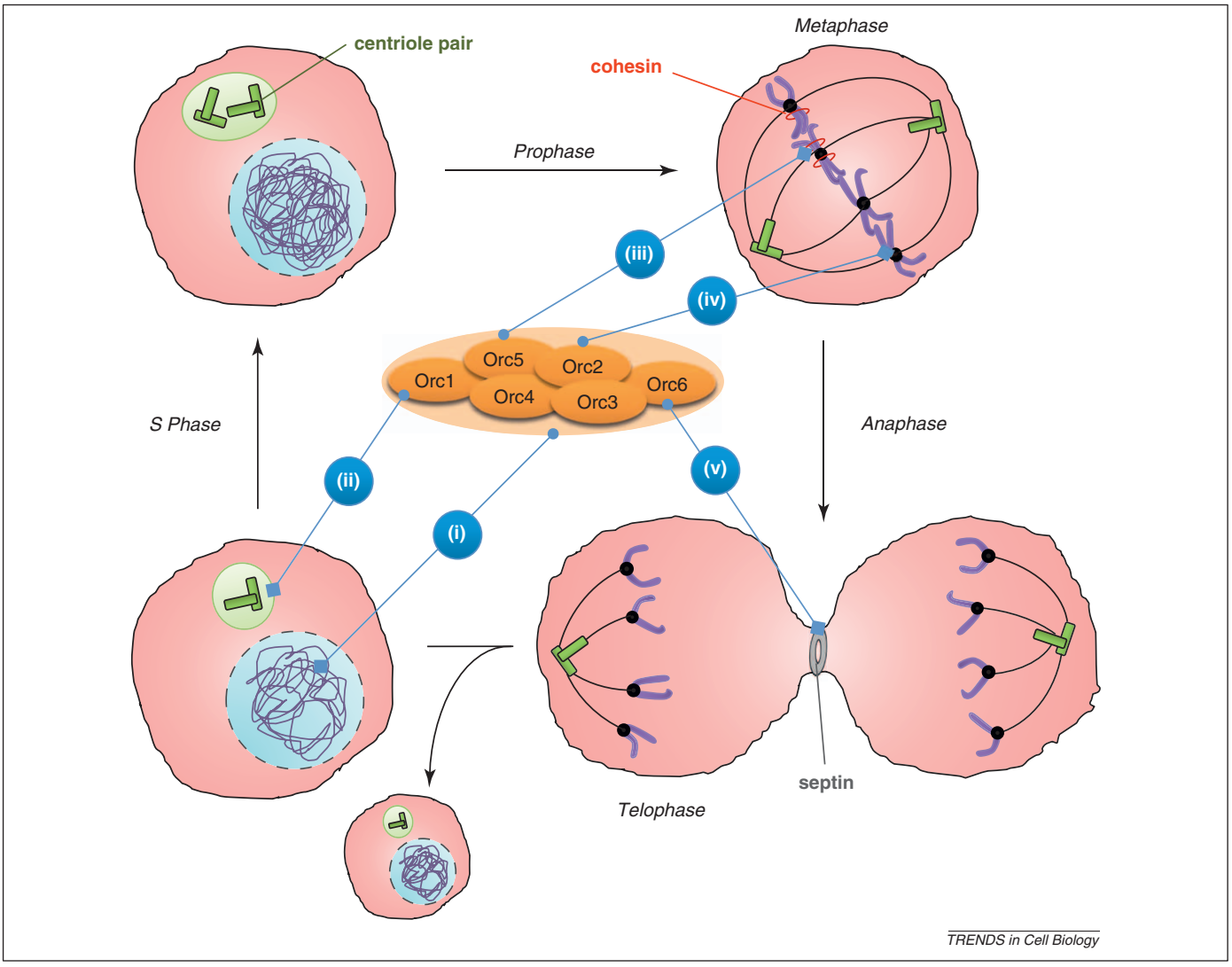

Figure 2. ORC participates in accurate chromosome segregation during mitosis. During S phase, ORC initiates DNA replication **(i)** and the Orc1-4 subunits localize at the centrosome where Orc1 regulates centrosome duplication **(ii)**. During metaphase, ORC facilitates sister chromatid cohesion through both cohesin-dependent **(iii)** and cohesin-independent pathways **(iv)**. During telophase, the cell undergoes cytokinesis and Orc6 has been implicated in this process through direct interaction with the filament-forming septin protein **(v)**.

of the cell cycle; chromosome segregation and cell division. In addition to these specific activities, initiator proteins affect progression of the cell cycle itself.

In *C. crescentus* the cell cycle proceeds by an asymmetric cell division, resulting in two distinct cell types: a stalked cell that is competent for DNA replication and a motile swarmer cell in which DNA replication is repressed (the swarmer cell must transition into a stalked cell to reinitiate the cell cycle) [33]. The *C. crescentus* cell cycle is governed by the oscillating presence of two crucial regulators, CtrA and GcrA [34]. During the swarmer cell to stalk cell transition, CtrA is degraded and *dnaA* is actively transcribed. At the peak of its expression, the DnaA protein displays distinct activities; initiation of DNA replication at the chromosome origin and transcriptional activation of the cell cycle regulator *gcrA* [26,35]. GcrA goes on to stimulate the transcription of several genes required for DNA replication and segregation, and directly activates the transcription of *ctrA* to reset the cell cycle. Thus, in *C. crescentus* the DNA replication initiator coordinates DNA synthesis with expression of a key cell-cycle regulator to drive the cell cycle forwards.

Many bacteria have the ability to alter their gene expression patterns from one cell cycle to the next in order to develop into specialized cell types. Recently, it was shown that DnaA coordinates DNA replication with spore development in *B. subtilis* (Figure 3). The process of sporulation involves a single cell developing into two distinct cell types, the mother cell and the forespore (which will mature into the dormant spore). Due to the cascade of signalling and transcription events that need to occur between and within the two cell types required for spore development, the process can be successfully completed only in a progenitor cell that has two fully replicated chromosomes, one destined for each cell type [36]. Many years ago it was found that there was a sensitive time within the cell cycle during which sporulation could be initiated [37]. Now it is clear that DnaA is responsible for this period of sensitivity by directly regulating the cell cycle-dependent transcriptional activation of the checkpoint protein Sda, a potent inhibitor

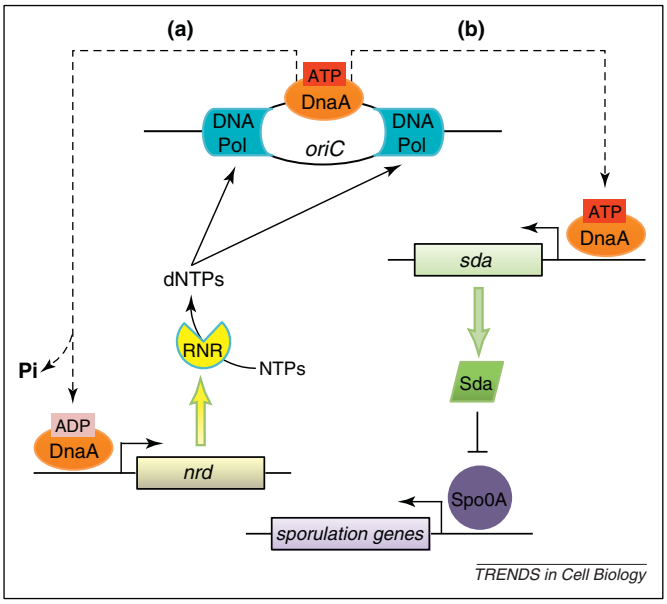

Figure 3. DnaA coordinates DNA replication initiation with cellular metabolism and development through transcriptional activation of target genes. **(a)** Following the initiation of DNA replication in *Escherichia coli,* DnaA is inactivated through ATP hydrolysis and transcription of *nrd* is driven by ADP-bound DnaA. Expression of *nrd* leads to the production of ribonuclease reductase (RNR), which synthesizes the dNTPs required for DNA replication. **(b)** Concomitant with the initiation of DNA replication in *Bacillus subtilis,* DnaA directly activates the transcription of *sda.* Expression of *sda* leads to the production of the Sda checkpoint protein that inhibits the activity of Spo0A, the master transcriptional regulator of sporulation.

of the master sporulation regulator, Spo0A (Figure 3) [38,39]. Using single-cell analysis it was observed that the peak in DnaA-dependent Sda expression is concomitant with DNA replication initiation. Importantly, mutations in regulatory factors that activate DnaA to initiate DNA replication also activate DnaA to stimulate *sda* transcription, suggesting that the ATP-bound form of DnaA is responsible for this regulation [40]. Taken together, these results indicate that DnaA inhibits spore development during DNA replication initiation to allow time for the requisite chromosome content to be achieved.

As a parallel with the function of DnaA regulating bacterial development, it has been suggested that Orc proteins play roles in the development of specialized eukaryotic cell types [41]. Cultured differentiated mouse hippocampus neurons express a subset of Orc proteins (Orc2–Orc5); because these cells are no longer proliferating, it suggests that the eukaryotic DNA replication initiator proteins are involved in processes other than DNA synthesis [42,43]. Consistent with this notion, the murine Orc2–Orc5 proteins display a distinctive non-nuclear localization pattern and are enriched in the membrane fraction of the postsynaptic dendritic compartment. Knockdown of Orc3 and Orc5 proteins reduced dendritic branch and spine formation, while expression of a mutant Orc4 protein enhanced the branching of dendritic arbors. In another study, it was found that *Drosophilia* Orc3 protein localizes at neuromuscular junctions and that an *orc3* mutant caused defects in neural cell proliferation [44,45]. Thus, Orc proteins appear to play important roles in the regulation of neural cell development, although the mechanisms by which they act are unclear. These unexpected roles for eukaryotic DNA replication initiator proteins open the provocative possibility that they could play novel roles in the development of other specialized cell types.

DNA replication initiator proteins as regulators of cellular metabolism

All of the interrelated cell cycle processes discussed thus far are energy intensive and must be coordinated with cell growth (i.e., the increase in cell mass). Cells must constantly monitor their nutritional status in order to gauge their capacity to proliferate. Evidence from studies in both bacteria and eukaryotes suggest that DNA replication initiator proteins play important roles by coordinating DNA replication with cellular metabolism, helping to ensure the availability of building blocks required for cell growth and cell cycle progression.

The connection between bacterial metabolism and DNA replication initiator proteins has been revealed through genome-wide microarray-based DNA binding assays and transcriptional profiling experiments. DnaA was found to bind the promoter regions of several metabolic genes and to regulate their transcription. The products of these genes are involved in a wide range of activities, including nucleotide biosynthesis, carbohydrate metabolism, iron homeostasis, amino acid biosynthesis and ribosome biogenesis [24–26,46]. DnaA regulation of the *nrd* operon encoding the enzyme ribonucleotide reductase (RNR), which catalyzes the final step in the synthesis of deoxyribonucleotides, is particularly intriguing because it has been observed in *B. subtilis*, *C. crescentus,* and *E. coli* [24–26,47].

Genetic and biochemical studies have begun addressing the molecular nature of *nrd* operon regulation, whereby DnaA stimulates transcription of *nrd* in a cell cycle-dependent manner (Figure 3). In *E. coli,* the concentration of ATP-bound DnaA increases before DNA replication and following initiation DnaA is stimulated to hydrolyse ATP, leading to the accumulation of ADP-bound DnaA within the cell [48]. Transcription of *nrd* is activated after DNA replication initiation and this activation requires binding sites recognized by ADP-bound DnaA within the *nrd* promoter [49,50]. Taken together, these observations suggest that DnaA is utilized to precisely regulate RNR expression in order to coordinate deoxyribonucleotide synthesis with DNA polymerization, thus ensuring the availability of substrates required for genome duplication.

Similarly, recent work has provided evidence for a link between DNA replication initiators and metabolic genes in eukaryotes. ChIP-chip analysis identified high-affinity ORC binding sites in *S. cerevisiae* and found that ORC preferentially localized within the open reading frames of many highly transcribed genes involved in the metabolism of various compounds, including amino acids, carboxylic acids and nitrogen [51]. ORC association at these high-affinity binding sites did not promote DNA replication initiation, supporting the idea that these sites are utilized for a distinct function unrelated to DNA synthesis. Many of the metabolic genes bound by ORC were located proximal to *bona fide* origins and specific origin activation increased the binding of ORC at a neighboring metabolic gene. Although more work is required to establish the role of ORC binding to these highly transcribed genes, the authors

speculated that these ORC binding sites might coordinate DNA replication with the expression of metabolic proteins required for proper cell growth. It will be highly informative to ascertain whether transcription of ORC-bound metabolic genes is activated by ORC.

We note that while the role of ORC as a transcriptional repressor is well established (e.g., through Sir1-mediated gene silencing [31]), there is little historic data indicating that ORC could act also as a transcriptional activator. Now, a recent study in the plant *Arabidopsis thaliana* has found that the Orc1 protein binds preferentially to methylated histones within the promoter region of target genes and acts as an effector protein to activate gene expression directly [52]. Further global gene expression experiments should reveal whether Orc proteins are widely used for positive transcription regulation and whether they act analogously to bacterial DNA replication initiator proteins to coordinate DNA replication with metabolic and/or cell cycle activities.

Perspectives

In this review, we have presented evidence documenting the roles of DNA replication initiator proteins outside of their well characterized activities in DNA synthesis. The data indicate that the functional homology between prokaryotic and eukaryotic DNA replication initiator proteins can be extended to include roles as important regulators linking DNA synthesis with chromosome segregation, cytokinesis, cell growth and cellular development. This conservation suggests that it is beneficial for cells to coordinate the initiation of DNA replication with multiple aspects of the cell cycle in order to maximize the likelihood of faithful genome replication and inheritance.

On the basis of work from several diverse model systems, it appears that the bacterial initiator proteins exert their influence on the cell through transcriptional regulation. One of the major outstanding questions regarding DnaA activity is how the protein mediates positive and/or negative transcriptional regulation at various genes. Sequence analysis shows that DnaA binding sites vary widely in their number (from a few to >10), location (upstream and/or downstream of promoters) and orientation (relative to the direction of transcription). Moreover, the DNA-binding activity of DnaA will be influenced by both its bound nucleotide and additional DNA-binding proteins recruited to specific promoters. This complexity has made it impossible to predict the mechanism(s) of transcriptional regulation by DnaA, although it is likely that negative regulation at some genes is mediated by a simple occlusion mechanism whereby DnaA binding inhibits promoter recognition by RNA polymerase. For the majority of cases, experimental details will need to be obtained for each promoter in order to determine the mechanisms of transcriptional regulation and the temporal link between gene expression and DnaA activity.

In contrast, eukaryotic initiator proteins appear to regulate the cell cycle using a more diverse set of mechanisms, including direct protein–protein interactions inside and outside the nucleus. We note that the bacterial DnaA protein is composed of a single polypeptide that must contain all of the activities required to initiate DNA replication, whereas eukaryotic ORC is composed of six distinct subunits. Therefore, single subunit initiator proteins might be limited in the number of possible regulatory mechanisms they can exploit, but the hexameric structure of ORC would facilitate the opportunity for individual subunits or subcomplexes to acquire specialized regulatory activities, which has been clearly observed for the C-terminal domain of Orc6. Furthermore, it is possible that splice variants of Orc proteins would engender myriad potentially diverse activities, and indeed novel variant forms of murine Orc1-3 derived from alternative RNA splicing have been reported [53].

Several additional studies have provided tantalizing indications of still further roles for DNA replication initiator proteins. Genome-wide analyses suggest that DnaA might regulate DNA recombination and repair, antibiotic production and protein secretion [24,26]. Similarly, a yeast two-hybrid interaction study using Orc proteins as bait identified interactions with a number of cell cycle regulators, including the Cdc28p phosphatase and the spindle assembly checkpoint protein Mad1p [54]. Thus, it seems likely that the full extent to which DNA replication initiator proteins are utilized throughout the cell cycle and during cellular development has yet to be realized.

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